SUMMARY

1. Citrate increases the rotation of the molybdate complexes of malic acid (as already observed by Auerbach & Krüger [1923]), of citramalic acid and of isocitric acid. The increase can amount to more than 100%. The magnitude of the effect under varying conditions is investigated.

2. The effect of citrate must be taken into account when the above acids are determined polarimetrically by the molybdate method. Isocitric acid concentrations calculated from polarimetric readings by previous investigators who were unaware of the citrate effect require revision.

3. Preliminary experiments show that the equilibrium mixture of isocitrate, *cis*-aconitate and citrate in the presence of liver or muscle aconitase contains 6·2% isocitrate, 4·3% *cis*-aconitate and 89·5% citrate (38°; *p*H 6·8; 0·025*M* phosphate buffer). The effect of *p*H is small between 6·8 and 7·4. $MgCl_2$ shifts the equilibrium in favour of citrate.

4. A modified polarimetric method is suggested for the determination of malic and isocitric acids, applicable to solutions containing citrate.

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Aerobic Oxidation of Aromatic Hydrocarbons in the Presence of Ascorbic Acid

THE REACTION WITH ANTHRACENE AND 3:4-BENZPYRENE

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It has long been known that oxidation of the aromatic hydrocarbons plays a fundamental part in their elimination from the animal body, but isolation of the products excreted has contributed little to knowledge of the initial introduction of oxygen into the molecule. This problem has assumed even greater importance recently in connexion with the fate of carcinogenic hydrocarbons in the animal body. Many such hydrocarbons are now known and, in most cases, introduction of oxygen into the molecule leads to considerable or complete loss of carcinogenic activity. Thus in these carcinogens oxidation is equivalent to 'detoxication' from the point of view of cancer induction.

It is well known that the feeding of naphthalene to rabbits leads to the production of cataract. The crystalline lens normally contains much ascorbic acid. Simultaneously with the development of opacity in the lens during cataract formation the ascorbic acid content falls to a very low value. It has also been reported that the administration of ascorbic acid to animals on a naphthalene diet inhibits cataract production. It must be admitted that the whole question of the relation of ascorbic acid to cataract production by naphthalene is still undecided, and there is no general agreement on the facts. However, these experiments suggested to Jorissen [1937] the desirability of testing the

action of ascorbic acid on naphthalene *in vitro*. In the aqueous acetone solution of naphthalene containing ascorbic acid, he detected the formation of an oxygen-containing derivative of naphthalene which he assumed to be a naphthol.

This observation has been studied in more detail in the present work and extended to other hydrocarbons. Evidence of oxidation of the hydrocarbon has been obtained with naphthalene, phenanthrene, anthracene, and with the carcinogenic hydrocarbons 3:4-benzpyrene, cholanthrene, and methylcholanthrene. Experiments on the identification of the products from anthracene and 3:4-benzpyrene are described.

EXPERIMENTAL

The technique was very simple. The hydrocarbon was dissolved in a suitable volume of 80 % acetone (B.D.H. Analar) and 2–4 times its weight of ascorbic acid added. The flasks, which were large enough to contain ample air, were stoppered and kept in the dark at room temperature (usually about 20°). Light was excluded to avoid photo-oxidation.

Anthracene

Examined after 2 weeks, solutions containing anthracene and ascorbic acid showed no obvious difference in colour when compared with similar control solutions containing no ascorbic acid. Water was added to the solution and the acetone removed under reduced pressure at a low temperature. The solid material which separated (mainly unchanged anthracene) was collected and well washed with water. This residue was heated with *N* NaOH and Zn dust. The filtrate from Zn and unchanged hydrocarbon was deep red. The extraction with alkali and Zn was repeated until the filtrates and washings were colourless. On aeration of the alkaline filtrate a brownish solid separated and the solution lost its deep red colour. The solution was made slightly acid and the precipitate collected and dried. Recrystallization from glacial acetic acid gave fine yellow needles, M.P. 279–280° (uncorr.), which did not depress the M.P. (279–280°) of authentic anthraquinone.

In solutions containing anthracene and ascorbic acid left for longer periods (2–3 months) crystals of anthraquinone were seen to form as long very pale yellow needles, which on removal and recrystallization had M.P. 279–280°. No anthraquinone could be detected in the control experiments.

3:4-Benzpyrene

This hydrocarbon invariably showed a most striking and characteristic colour change. Benzpyrene in aqueous acetone kept in the dark maintains its pale yellow-green colour even after 5 years. In the presence of ascorbic acid the solution rapidly becomes deep yellow, then orange and finally ruby red. The change is detectable in a few hours, and after standing overnight it is quite obvious.

Isolation of benzpyrene quinones. After removal of the acetone under reduced pressure the precipitated solid was collected by filtration. This solid consisted mainly of unchanged hydrocarbon impregnated with some coloured material which gave the mass a magenta colour. Treatment of this product with 2*N* NaOH in the cold gave a dark alkaline solution with a strong green fluorescence. Acidification of this solution led to its decolorization with precipitation of a small quantity of red flocculent material. The nature of this material is discussed later.

The oxidation product was repeatedly leached with hot 2*N* NaOH until the extracts had lost all the green fluorescence and were practically colourless. Boiling the extracted residue with more 2*N* NaOH and Zn dust led to a red-brown vat. Unchanged hydrocarbon was removed by filtration and the alkaline solution aerated. A red precipitate separated and the solution became very pale green. After acidification the precipitate was collected and dried, when it was obtained as a brick-red powder. The yield was low—*ca.* 10 mg. from 100 mg. of benzpyrene. The melting-point after recrystallization from xylene was 230°, not depressed by admixture with a specimen of 3:4-benzpyrene quinone M.P. 230° [Cook & Hewett, 1933].

Vollmann, Becker, Corell & Streeck [1937] showed that the quinone described by Cook & Hewett is a mixture of 5:8- and 5:10-quinone. Separation was achieved by conversion of the mixture of quinones to the leucodiacetates and fractional crystallization. The 5:10-quinone crystallizes in golden orange needles M.P. 295° and dissolves in concentrated H_2SO_4 to give a carmine red solution. The 5:8-quinone forms red needles M.P. 245° and its solution in conc. H_2SO_4 is olive brown.

The product from ascorbic acid oxidation of benzpyrene gave a solution in conc. H_2SO_4 intermediate in colour between solutions of the pure quinones and indistinguishable from that of a solution of the mixed quinones of Cook & Hewett.

Other products of the oxidation. As mentioned above, some material is extractable from the oxidation product by alkali alone. Since this material can be apparently completely removed before the quinones are extracted by vat formation, it is not quinonoid but presumably phenolic in nature. On precipitation with acid from its alkaline solution it is red and flocculent, but on collection and drying it darkens. All efforts at purification of this material have so far been unsuccessful and have yielded nothing but further small quantities of quinone.

Colorimetric measurements on the oxidation product. These were made by means of a Hilger Photoelectric Absorptiometer using Ilford 'Spectrum' filters. The colour developed in mixtures of benzpyrene and ascorbic acid solution was measured and analysed by using the filters at intervals during 58 days. A typical result is shown in Fig. 1, in

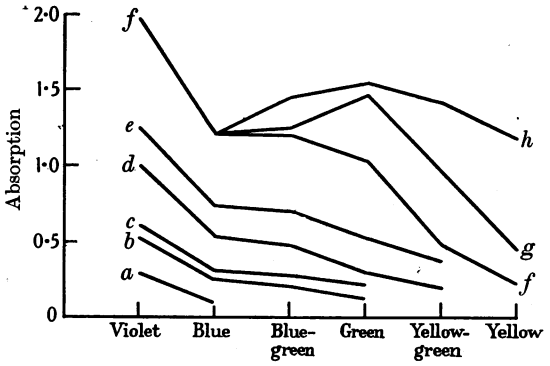


Fig. 1. Colour development in a solution of 3:4-benzpyrene during aerobic oxidation in the presence of ascorbic acid. $a=27$, $b=50$, $c=74$, $d=98$, $e=144$, $f=214$, $g=339$ hr. $h=58$ days.

which are plotted the values obtained by measurement of a solution initially containing 50 mg. benzpyrene and 200 mg. ascorbic acid. Similar measurements were carried out on pure specimens of the 5:8- and 5:10-quinone and on the mixture prepared by chromic acid oxidation of the hydrocarbon (Table 1). Attention may be directed to has fallen by 20 %. This change is not shown by the 5:10-quinone where the absorptions of saturated solutions in pure acetone or in 80 % aqueous acetone are practically indistinguishable.

Comparison of the values shown in the table with those for the ascorbic acid-hydrocarbon experiment given in Fig. 1 indicate that the colour developed in the latter is mainly due to formation of the quinones but that there is also some other chromogen present. In the later curves in the figure the absorption towards the red end of the spectrum is greater than can be accounted for by assuming a saturated solution of the mixed quinones, even if the more chromogenic 5:8-compound predominated.

The influence of various factors on the oxidation. By making use of the rapid development of colour with benzpyrene it was possible to examine the influence of various factors on the speed of the reaction. At temperatures of 30–40° the reaction proceeded more rapidly than at 20–25°, but measurements of the colour produced showed that the reaction at the lower temperatures, although initially slower, finally developed more colour. This is to be expected since the rate of destruction of the ascorbic acid is much greater at the higher temperature range. The rate of reaction was not appreciably increased by vigorous shaking in air or O_2, but the reaction is completely inhibited if O_2 is excluded. Mixtures of ascorbic acid and benzpyrene in aqueous acetone in sealed tubes from which all air has been removed or replaced by N_2 remain colourless for months if they are not exposed to

Table 1. *Absorption measurements on quinones*

Filter ...	V.	B.	B.-G.	G.	Y.-G.	Y.	O.	R.
5:10-Quinone (100 % acetone)	∞	1·07	0·47	0·12	—	—	—	—
5:10-Quinone (80 % acetone)	∞	1·03	0·47	0·11	—	—	—	—
5:10-Quinone (80 % acetone) + ascorbic acid	∞	1·20	1·10	0·65	0·20	—	—	—
5:8-Quinone (100 % acetone)	∞	1·22	1·22	1·06	0·17	—	—	—
5:8-Quinone (80 % acetone)	∞	1·18	1·20	1·50	0·48	—	—	—
5:8-Quinone (80 % acetone) + ascorbic acid	∞	1·23	1·28	1·55	1·15	0·53	0·185	0·06
Mixed quinone in 80 % acetone	∞	1·28	1·28	1·05	0·30	—	—	—

The values = absorption coefficient. Cell depth, 1·0 cm. Concentration—saturated at 15° C.

two points. One is the increase in intensity of the colour of the quinone solutions, particularly towards the red end of the spectrum, when ascorbic acid is present in their solutions. This is especially marked in the case of the 5:8-quinone where the absorption, for example, in the yellow-green is increased from 0·48 to 1·15 on adding ascorbic acid and allowing to stand for several days. The second point is that on diluting the 100 % acetone solution of the 5:8-quinone with water in order to obtain an 80 % solution there is an increase in the absorption intensity although the concentration of the quinone

bright light. On opening the tubes the usual sequence of colour changes takes place.

Since it appeared probable that the autoxidation of the ascorbic acid played an essential part in the mechanism of the oxidation, the effect of copper, cyanide, metaphosphoric acid, and H_2O_2 on the reaction was tried.

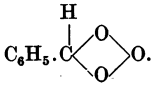
Small traces of cupric ions (1–2 μg./ml.) either had no effect or, occasionally, produced a slight acceleration of the reaction. $M/100$ KCN or 5 % HPO_3, however, completely inhibited the reaction. In view of the possibility that the oxidation of the

hydrocarbon might be due to H_2O_2 formed during autoxidation of the ascorbic acid, experiments were carried out in which H_2O_2 was added to the hydrocarbon-ascorbic acid mixture, and also some in which H_2O_2 alone without ascorbic acid was employed. The addition of the peroxide was without effect on the reaction when both [illegible] and ascorbic acid were present in the solution. Alone, the H_2O_2 did not oxidize the hydrocarbon.

These results indicated that the enediol grouping of ascorbic acid was the essential structural requirement for bringing about the oxidation of the hydrocarbon. Support for this view was obtained in experiments in which ascorbic acid was replaced by dihydroxymaleic acid. Exactly similar oxidations of hydrocarbons were obtained by the use of this acid in aqueous acetone solution.

DISCUSSION

The oxidation of aromatic hydrocarbons in the presence of substances undergoing autoxidation is well known. In a study of the inhibitory action of anthracene on the autoxidation of benzaldehyde Bäckström & Beatty [1931] have shown that the inhibitory action is connected with an induced oxidation of the inhibitor. The primary oxidation product is anthranol which is autoxidizable and reacts with oxygen to give a peroxide which is slowly decomposed with the formation of the final reaction product, anthraquinone. The course of the reaction indicated that the initial oxidation of the hydrocarbon is the result of a reaction with a peroxide of benzaldehyde of the structure



Wasley & Rusch [1942] showed that the autoxidation of benzaldehyde and of heptaldehyde was inhibited by anthracene, 3:4-benzpyrene, 1:2:5:6-dibenzanthracene, and 20-methylcholanthrene. In the case of benzpyrene, measurements of absorption spectra indicated that the inhibition involved the oxidation of the hydrocarbon to one or more quinones.

It seems probable that a similar mechanism underlies the reaction described in the present paper. The autoxidation of ascorbic acid may be assumed to involve the formation of a peroxide. If this peroxide oxidizes anthracene to anthranol and 3:4-benzpyrene to 5-hydroxy-3:4-benzpyrene as the initial products, the formation of anthraquinone and a mixture of benzpyrene quinones together with some monohydroxybenzpyrene is readily understood.

Whether such a mechanism plays any part in the *in vivo* oxidation and elimination of aromatic hydrocarbons remains undecided. Experiments on this point, and investigations of the possibility that aromatic hydrocarbons interfere with the normal utilization of ascorbic acid in the animal body, are in progress.

SUMMARY

Oxygen oxidizes aromatic hydrocarbons in aqueous acetone solutions containing ascorbic acid. Anthracene is oxidized to 9:10-anthraquinone and 3:4-benzpyrene yields a mixture of 3:4-benzpyrene-5:8-quinone and 3:4-benzpyrene-5:10-quinone. The oxidizing agent appears to be one of the products formed during autoxidation of ascorbic acid and is possibly dehydroascorbic acid or a peroxide thereof. The mechanism seems to be similar to that which occurs during the inhibition of the autoxidation of aldehydes by aromatic hydrocarbons.

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